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VEGF Receptor Tyrosine Kinases

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<b>13. ABSTRACT (Maximum 200 Words)</b> Angiogenesis plays a fundamental role in solid tumor growth and metastasis. The vasculature is for the most part quiescent in the adult, so it follows that anti-angiogenic therapies are an attractive method of targeting tumorigenesis. Thus, the purpose of this study was to study the basic mechanisms behind vascular development with the goal of discovering novel proteins involved in angiogenic signal transduction pathways. This project both informs the understanding of angiogenesis and has the potential benefit of identifying novel, specific targets for anti-angiogenic therapy.  Two families of endothelial receptor tyrosine kinases play an essential role in angiogenesis: VEGF and Tie. In order to find new members of this signaling pathway, we performed a yeast two-hybrid screen of a human fetal heart library using the VEGFR1 kinase domain as bait. We discovered that VEGFR1 associated with SOCS2 in a specific, kinase-dependent manner. SOCS2 is a member of the SOCS family of cytoplasmic signaling proteins that share homology in their C-terminal SOCS-box. Interestingly, other members of the family demonstrate a negative regulatory effect on cytokine signaling. Thus, we hypothesized that SOCS2 has a role on VEGF growth factor signaling through the VEGFR1 receptor.				
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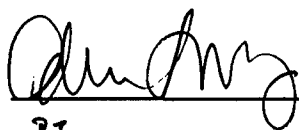
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## Introduction

Angiogenesis plays a fundamental role in solid tumor growth and metastasis. The vasculature is for the most part quiescent in the adult, so it follows that anti-angiogenic therapies are an attractive method of targeting tumorigenesis. Thus, the purpose of this study was to study the basic mechanisms behind vascular development with the goal of discovering novel proteins involved in angiogenic signal transduction pathways. This project both informs the understanding of angiogenesis and has the potential benefit of identifying novel, specific targets for anti-angiogenic therapy.

Two families of endothelial receptor tyrosine kinases play an essential role in angiogenesis: VEGF and Tie. In order to find new members of this signaling pathway, we performed a yeast two-hybrid screen of a human fetal heart library using the VEGFR1 kinase domain as bait. We discovered that VEGFR1 associated with SOCS2 in a specific, kinase-dependent manner. SOCS2 is a member of the SOCS family of cytoplasmic signaling proteins that share homology in their C-terminal "SOCS-box". Interestingly, other members of the family demonstrate a negative regulatory effect on cytokine signaling. Thus, we hypothesized that SOCS2 has a role on VEGF growth factor signaling through the VEGFR1 receptor.

## Body

This work differs from that originally outlined in the approved statement of work. The cDNA RDA screen proposed in my original statement was performed to completion (see annual report, 1999). Unfortunately this screen as performed did not yield any novel proteins which were transcriptionally regulated by VEGF, making impossible to go further with that project. Reasons for the failure of this screen could include: 1. looking at the wrong timepoints for transcriptional regulation, 2. using a cell line that for whatever reason did not induce protein transcription in the way that an endothelial cell in vivo would, or indeed 3. the lack of a novel transcriptionally regulated protein to be found. Therefore, the following summary focuses on our study of an earlier stage of VEGF-induced angiogenesis, signal transduction. The signal transduction pathways of angiogenesis have not been completely elucidated and are equally important to the overall understanding of tumor angiogenesis.

The relationship between angiogenesis and solid tumor progression has been well established. Therefore, an understanding of the basic mechanisms of angiogenesis benefits not only the vernacular but may lead to novel cancer-targeting therapeutics.

Receptor Tyrosine Kinases (RTKs) and their cognate ligands play important roles in vascular growth and development . We performed a screen for novel members of the VEGF signaling pathway, to answer two important questions:

- What signal transduction events occur post growth-factor stimulation of endothelial RTKs?
- How do they affect specific aspects of the growth and maintenance of the vascular network?

The Flt-1 kinase domain was used as bait in a yeast-two hybrid screen of a human fetal heart library (in collaboration with M. Blonar, Bristol-Myers Squibb). This screen demonstrated a novel association between Flt-1 and SOCS2, a SH2-domain containing cytoplasmic protein.

The SOCS family, so named for their function as "Suppressors Of Cytokine Signaling" display a homology via their C-terminal "SOCS box". The SOCS box has a characteristic N-terminal BC box and C-terminal L/P rich sequence, and its function has been under debate: it is either a protein stabilizer or a targeting protein for protein degradation. Several members of this family have been shown to function as negative regulators of signal transduction. They affect JAK/STAT signaling via the SH2 domain and an N terminal kinase inhibitory region. Thus, our hypothesis is that SOCS2 modulates VEGF-induced angiogenesis by binding to the RTK via its SH2 domain.

Further bait testing demonstrated a kinase- or phosphotyrosine-dependent association between Flt-1, Flk-1 and Tie-2 and SOCS2 (Fig. 1). SOCS2 was tested for association with baits consisting of the kinase domains of the four main endothelial RTKs. KR denotes a point mutation in the ATP-binding site rendering the bait protein kinase-deficient.

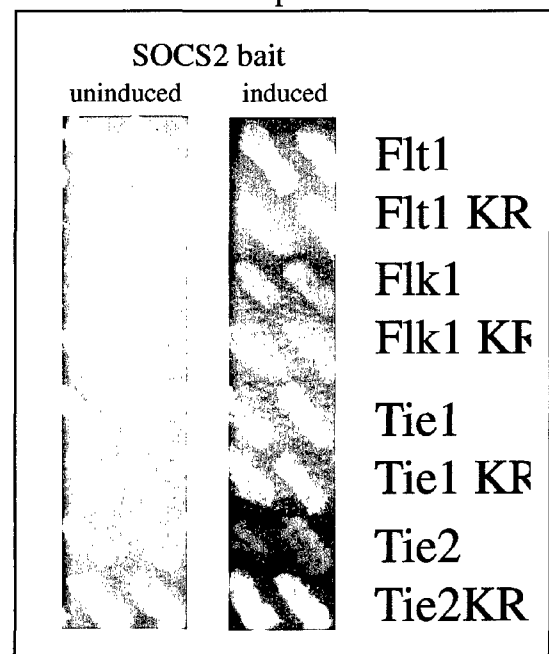


Figure 1

Using a GST-pulldown assay, Flt-1 kinase and Flk-1 kinase both associate with the SH2 domain of SOCS2 in a kinase-dependent manner (Figs. 2-4). In figure 3, equal amounts of wild type (wt) and kinase-inactive (KR) GST-Flt-1 kinase were bound to beads [GST, PY99 blots]. Flag-SOCS2 bound exclusively to the wt and not the KR. Flag-SOCS2RK, with a point mutant in its SH2 domain, did not bind to either wt or KR GST-Flt-1. [Flag blot] The association of PLC $\gamma$  and Grb2 with wt GST-Flt-1 was not affected by overexpression of SOCS2 [PLC $\gamma$ , Grb2 blots]. In figure 4, the same is seen with GST-Flk-1 kinase.

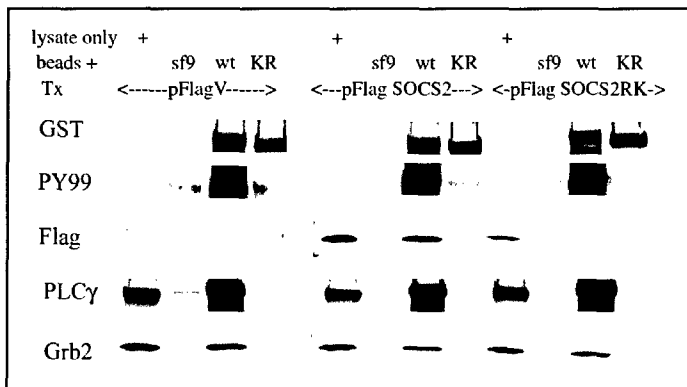
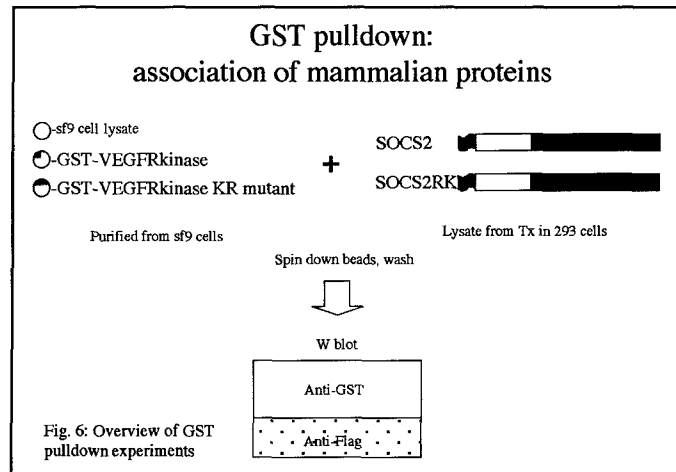


Figure 3: GST-Flt kinase association w/ SOCS2

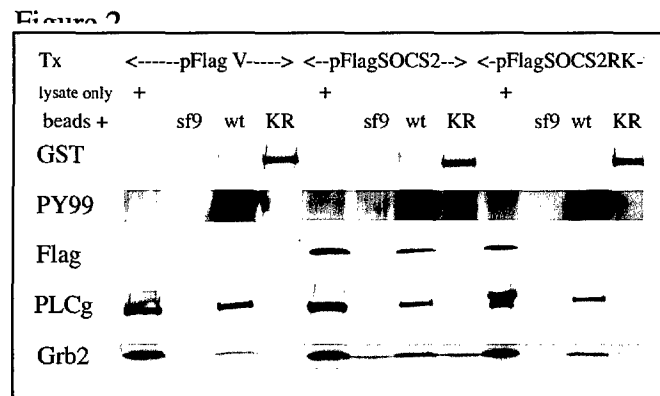
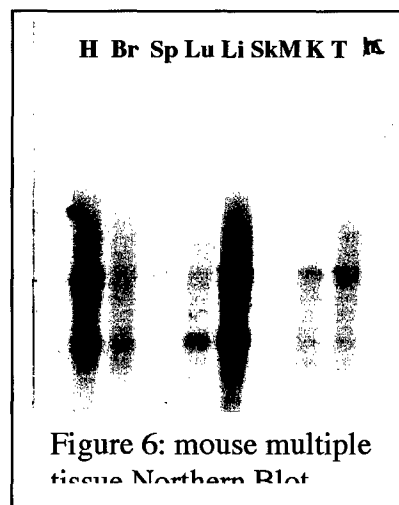
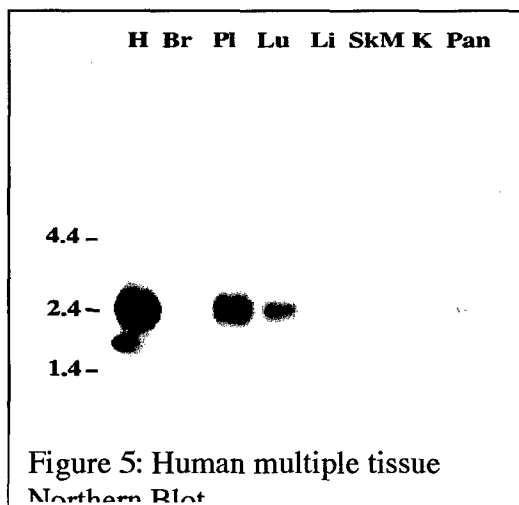


Figure 4: GST-Flk kinase association w/ SOCS2

Northern blotting demonstrated SOCS2 expression in highly vascularized adult tissues and at various developmental stages (Fig. 5-7). Northern blots were probed with either murine or human SOCS2 probe. H=heart, Br=brain, Pl=placenta, Lu=lung, Li=Liver, SkM=skeletal muscle, K=kidney, Pan=pancreas, Sp=spleen, T=testes. Mouse developmental blot contains poly A RNA from embryonic day 7, 11, 15, and 17.



Whole-mount *in situ* hybridization of mouse lung reveals SOCS2 mRNA expression in the mesenchyme surrounding developing lung buds at day 12.5, consistent with an area of developing vasculature (Fig. 8)

Immunohistochemistry demonstrates SOCS2 protein expression in the endothelium of placental blood vessels, a site of active angiogenesis (Fig. 9). This polyclonal antibody against human SOCS2 was generated by our laboratory and purified against a His-SOCS2 affinity column.

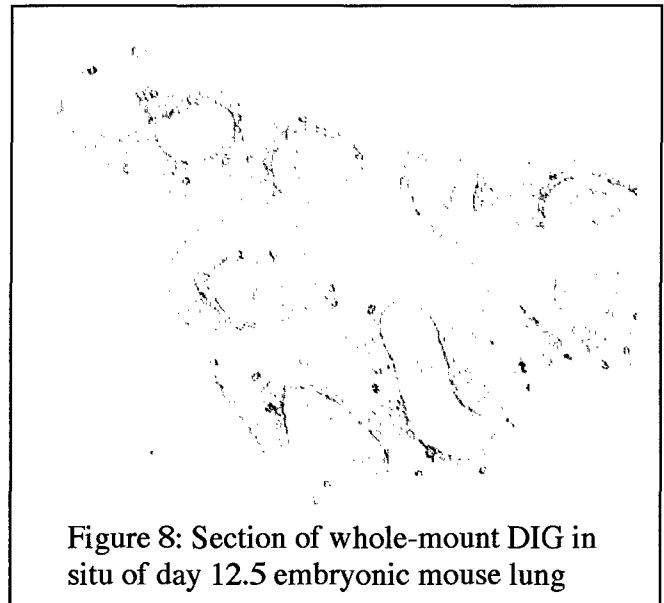


Figure 8: Section of whole-mount DIG in situ of day 12.5 embryonic mouse lung

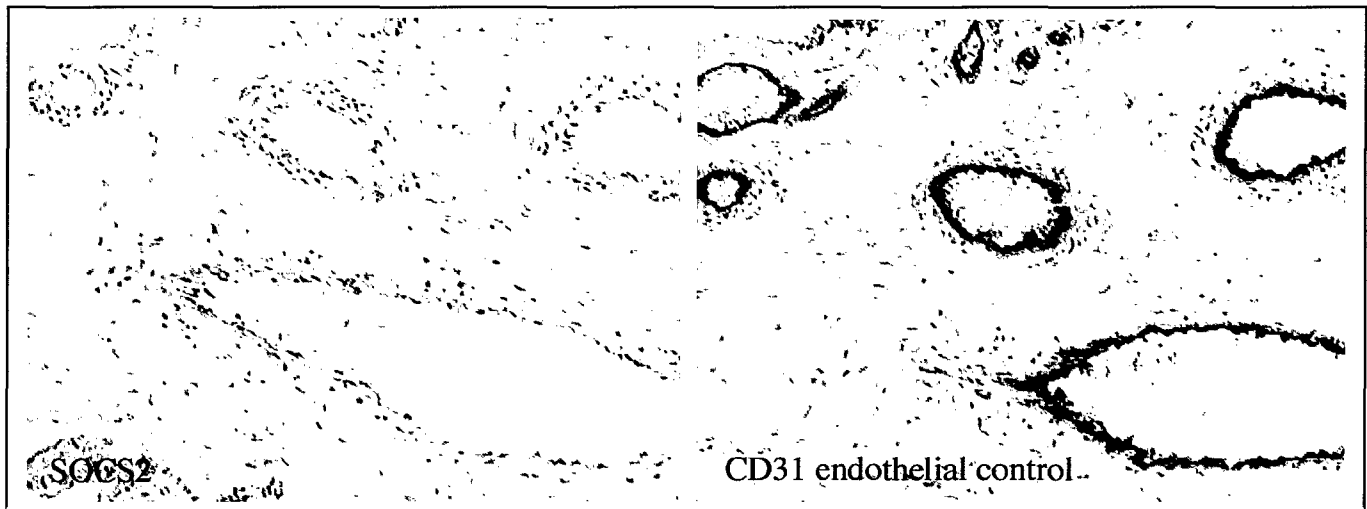


Figure 9: Immunohistochemical staining of SOCS2 in placental blood vessels

In summary,

1. The association of VEGFR1 and VEGFR2 with SOCS2 has been demonstrated in both the yeast two-hybrid system and a GST-pulldown system.
2. This association is dependent upon the presence of both a functional kinase domain on the receptor and an intact SH2 domain in SOCS2.
3. Expression studies have detected SOCS2 mRNA in highly vascularized tissues and SOCS2 protein in the endothelium of the placenta, adding weight to the hypothesis that the *in vitro* association between SOCS2 and VEGF receptors occurs *in vivo*.

Future directions include:

1. Determine how VEGF stimulation regulates SOCS2 association with VEGF receptors in endothelial cells.
2. Determine function of SOCS2 interaction with VEGFR in angiogenesis

## Key Research Accomplishments:

- Demonstrated via the yeast two-hybrid system a novel association between VEGFR1 and VEGFR2 with SOCS2.
- Demonstrated via a GST-pulldown system the novel association between VEGFR1 and VEGFR2 with SOCS2.
- Demonstrated that this association is dependent upon the presence of both a functional kinase domain on the receptor and an intact SH2 domain in SOCS2.
- Demonstrated that SOCS2 mRNA is expressed in highly vascularized tissues, and SOCS2 protein is expressed in the endothelium of the placenta.

## Reportable Outcomes:

### Abstracts and Presentations:

Poster Presentation, "Role of SOCS2 in VEGF and Tie-mediated angiogenesis",  
**Era of Hope Meeting, AMRMC Breast Cancer Research Program**, June 2000.

Poster Presentation, "Novel Association between Flt-1, Flk-1, Tie2 and SOCS2 in the yeast two-hybrid system", **American Society for Cell Biology Annual Meeting**, December 1999

Development of a replication-deficient Adenovirus which overexpresses SOCS2 wild-type protein.

## Conclusions:

We have clearly demonstrated a novel interaction between the VEGFR1 and VEGFR2 receptors with the SH2-domain containing protein SOCS2. This interaction occurs in both the yeast two hybrid system and the GST-pulldown system with mammalian proteins, and is dependent upon both a functional kinase domain and the presence of an intact SH2 domain. SOCS2 is present in the endothelium, so it would follow that this interaction has a functional significance *in vivo*. Further experiments will attempt to elucidate the function of this interaction *in vivo*.



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